Article

Mitochondrial abnormalities in iPSC-derived motor neurons from patients with riboflavin transporter deficiency

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Abstract: Riboflavin transporter deficiency (RTD) is a childhood-onset neurodegenerative disorder characterized by sensorineural deafness and motor neuron degeneration. Since riboflavin plays key functions in biological oxidation-reduction reactions, energy metabolism pathways involving flavoproteins are affected in RTD. We recently generated iPSC lines from affected individuals as an *in vitro* model of the disease and documented mitochondrial impairment in these cells dramatically impacting cell redox status. In the present work, we extend our study to motor neurons (MNs), *i.e.,* the cell type mostly affected in patients with RTD. Altered intracellular distribution of mitochondria was detected by confocal microscopic analysis, following immunofluorescence for superoxide dismutase 2 (SOD2), as a dual mitochondrial and antioxidant marker, and βIII Tubulin, as neuronal marker. We demonstrate significantly lower SOD2 levels in RTD MNs, as compared to their healthy counterparts. Mitochondrial ultrastructural abnormalities were also assessed by Focused Ion Beam/Scanning Electron Microscopy. Moreover, we investigated the effects of combination treatment using riboflavin and N-acetylcysteine, which is a widely employed antioxidant. Overall, our findings further support the potential of patient specific RTD models and provide evidence of mitochondrial alterations in RTD-related iPSC-derived MNs, emphasizing oxidative stress involvement in this rare disease. We also provide new clues for possible therapeutic strategies, aimed at correcting mitochondrial defects, based on the use of antioxidants.

Keywords: Riboflavin transporter deficiency; motor neurons; mitochondria; energy metabolism; electron microscopy; antioxidants; SOD2; oxidative stress; neurodegeneration.

1. Introduction

Riboflavin transporter deficiency (RTD), formerly known as Brown-Vialetto-Van Laere syndrome, is a rare autosomal-recessive motor neuron disease. Typical clinical features include peripheral and cranial neuropathy, muscle weakness, sensory loss, diaphragmatic paralysis and respiratory insufficiency, and multiple cranial nerve deficits such as sensorineural hearing loss, bulbar symptoms, and loss of vision due to optic atrophy [1,2]. Children affected with RTD show different phenotypes with variable prognosis, often influenced by the initiation of riboflavin (RF) treatment. In fact, some young patients treated with high-dose-RF supplementation show moderately improved muscle strength, motor function, respiration, hearing and vision [3-5]. Novel approaches based on combined RF/antioxidant supplementation are likely to be even more efficacious, since symptoms are only partially reversed by the sole RF treatment.

In the last decade, mutations in human RF transporter genes *SLC52A2* (encoding RFVT2) and *SLC52A3* (encoding RFVT3) were demonstrated as causative factors for RTD [5, 6]. RF represents an indispensable nutrient for human health, and its reduced intracellular availability compromises several vital processes, impacting the control of energy balance, particularly those depending on a proper redox status [7, 8]. Mitochondria, major organelles responsible for lipid and reactive oxygen species (ROS) metabolism, are therefore likely to be impaired in their integrity and bioenergetics in RTD syndrome.

Since *in vivo* models recapitulating symptoms and progression of RTD are so far lacking, we recently took advantage of induced pluripotent stem cell (iPSC) technology to reproduce the pathology in a patient-specific cellular model. We generated iPSCs from fibroblasts obtained from skin biopsies of patients carrying different mutations in *SLC52A2* gene to characterize their phenotype from a morpho-functional viewpoint. Specifically, we documented mitochondrial abnormalities, involving shape, number, and intracellular distribution. Also, we demonstrated redox imbalance, resulting from overproduction of superoxide anion, accompanied by abnormal mitochondrial polarization state. Moreover, patients' iPSCs showed altered expression of ROS-scavenging systems [9], encouraging the use of antioxidants for RTD treatment.

These results on patient-specific iPSCs, providing mechanistic insights into the disease pathogenesis to be used as a basis for innovative therapeutic approaches, prompted us to extend our study to motor neurons (MNs), i.e., the cell type mostly affected in patients with RTD. In the present investigation, we focus on mitochondrial morpho-functional features, studying their intracellular distribution and ultrastructural features, by confocal microscopy and Focused Ion Beam/Scanning Electron Microscopy, respectively. We performed immunofluorescence analysis of superoxide dismutase 2 (SOD2), as a dual mitochondrial and antioxidant marker, and of βIII Tubulin, as a neuronal marker, in combination with ultrastructural examination of the organelles, to shed light onto the role of oxidative stress in this neurodegenerative pathology. Moreover, we investigated the effects of a combined treatment employing RF and the antioxidant molecule N-acetylcysteine (NAC), to explore possible amelioration of neuronal phenotype associated to RTD involving mitochondrial compartment.

2. Materials and Methods

2.1. Derivation of iPSCs and differentiation into MNs

Human iPSCs were derived from fibroblasts of healthy subjects and two RTD patients carrying *SLC52A2* gene mutations (c.155C>T and c.935T>C; c.155C>T and c.1255G>A) by using the nonintegrating episomal technology (Epi Episomal iPSC Reprogramming Kit, A15960, ThermoFisher Scientific, Waltham, MA, USA). The identified mutations were confirmed after cell reprogramming, by Sanger sequencing on DNA extracted from RTD iPSC pellets, as previously reported [9]. Healthy and patient-specific iPSCs were then successfully differentiated into MNs, according to Corti and coll. [10].

2.2. Immunofluorescence and confocal microscopy

For confocal analysis, cells underwent our previously described double immunofluorescence protocol [9], using rabbit polyclonal antibody against mitochondrial superoxide dismutase 2 (anti-SOD2, ab13533, Abcam) and mouse monoclonal antibody to neuronal marker β III-tubulin (T8578, Sigma-Aldrich), as primary antibodies. Cells were then incubated with the appropriate secondary antibodies, conjugated with Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen, Carlsbad, CA, USA). Nuclei were stained with 1μg/ml Hoechst (33342, Invitrogen) and slides were observed in a Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany). Random images for statistical analysis purposes were captured by Leica Application Suite software and representative images were composed in an Adobe Photoshop CS6 format (Adobe Systems Inc., San Jose, CA, USA).

2.3. Electron microscopy

Ctrl and RTD MNs were plated in a chamber slide (Lab-Tek II Chamber Slide System, ThermoFisher Scientific). Some samples were treated with $1\mu M$ RF (R9504, Merck KGaA, Germany) and $100\mu M$ N-acetylcysteine amide (NAC) (A0737, Merck KGaA, Germany) overnight.

Treated and untreated cells were fixed and embedded in epoxy resin according to Colasuonno and coll. [11]. Electron microscopic observations and analyses were performed by a DualBeam Focused Ion Beam/Scanning Electron Microscope (FIB/SEM, Helios Nanolab, FEI, Hillsboro, OR, USA). Random images for statistical analysis purposes were electronically captured. Representative images were composed in an Adobe Photoshop CS6 format.

2.4. Statistical analysis

ImageJ (NIH) software was used to quantify immunofluorescence intensity while statistical analysis was performed using Prism software (GraphPad Software, Inc., La Jolla, CA, USA). A minimum of 300 cells/sample per experiment, were analyzed for fluorescence intensity levels.

For mitochondrial quantitative analyses, 3 samples/cell culture type and 10 cells/sample per experiment, were examined, by manually counting regular and altered mitochondria of Ctrl and RTD MNs and their percentage was calculated.

The results are presented as means ± SD of n ≥ 3 independent experiments. A *p* value of 0.05 or less was considered as statistically significant.

3. Results

3.1. Impaired SOD2 distribution and levels are associated with altered morphology of RTD neurons

Confocal analyses of RTD and Ctrl iPSCs-derived MNs were performed after immunofluorescence, using SOD2, as a dual marker for mitochondrial number and for antioxidant response, and βIII-tubulin (βIII-TUB) as a neuronal differentiation marker. While the distribution of βIII-TUB in Ctrl cells highlights long neuritic processes, forming an intricate network, in RTD MNs this neuronal marker reveals the presence of shorter neurites (Fig. 1, red signal). Such morphological changes are accompanied by reduced immunoreactivity to the mitochondrial marker SOD2 (Fig. 1, green signal in confocal images). Accordingly, quantitative analysis demonstrates significantly lower SOD2 expression levels in RTD MNs, as compared to their healthy counterpart (Fig. 1 (e)).
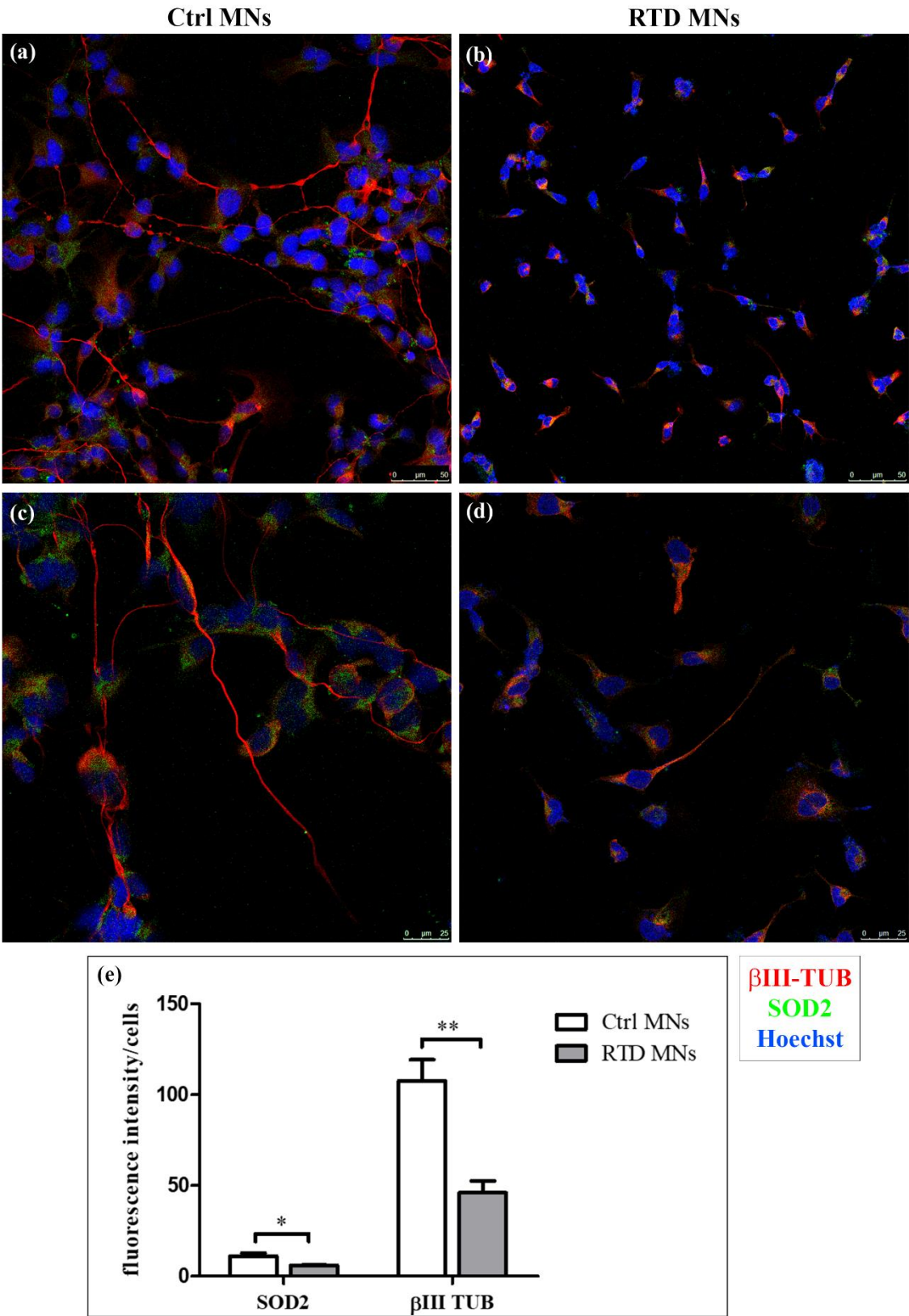
Figure 1. Confocal analysis of RTD vs. Ctrl MNs. **(a-d)** Immunofluorescence images showing the distribution of βIII TUB (red) and SOD2 (green). Nuclei are stained with Hoechst (blue). **(e)** Significantly lower levels of the mitochondrial marker SOD2 are detected in diseased MNs (*$p \leq 0.05$ vs*. Ctrl MNs).

3.2. Aberrant mitochondrial ultrastructure in RTD neurons is rescued by RF/NAC treatment

Data from confocal analyses prompted us to investigate mitochondrial features, as altered enzyme content likely reflects morphological changes. Indeed, FIB/SEM analyses of differentiated MNs highlights profound mitochondrial abnormalities, associated to RTD phenotype. These organelles appear swollen, with disrupted *cristae* (Fig. 2 (b), (d)), as opposed to mitochondria of Ctrl MNs, showing regular morphology (Fig. 2(a), (c)). Statistical analysis to evaluate the occurrence of altered mitochondria demonstrates the presence of a significantly higher number of damaged mitochondria in RTD *vs.* Ctrl MNs (Fig 2. (i)). Moreover, while Ctrl cells displayed several lysosomes and autophagosomes, fewer of these organelles were present in RTD MNs.

We also investigated at the ultrastructural level whether N-acetylcysteine (NAC), a glutathione (GSH) precursor, could exert a protective effect when administered with RF. As shown by electron microscopic images (Fig. 2 (e-h)), this combined treatment restores normal mitochondrial morphology in RTD MNs. In fact, statistical analysis demonstrates a significant decrease (**$p \leq 0.01$) of the number of damaged mitochondria in patients' MNs after RF+NAC treatment (Fig. 2 (i)).
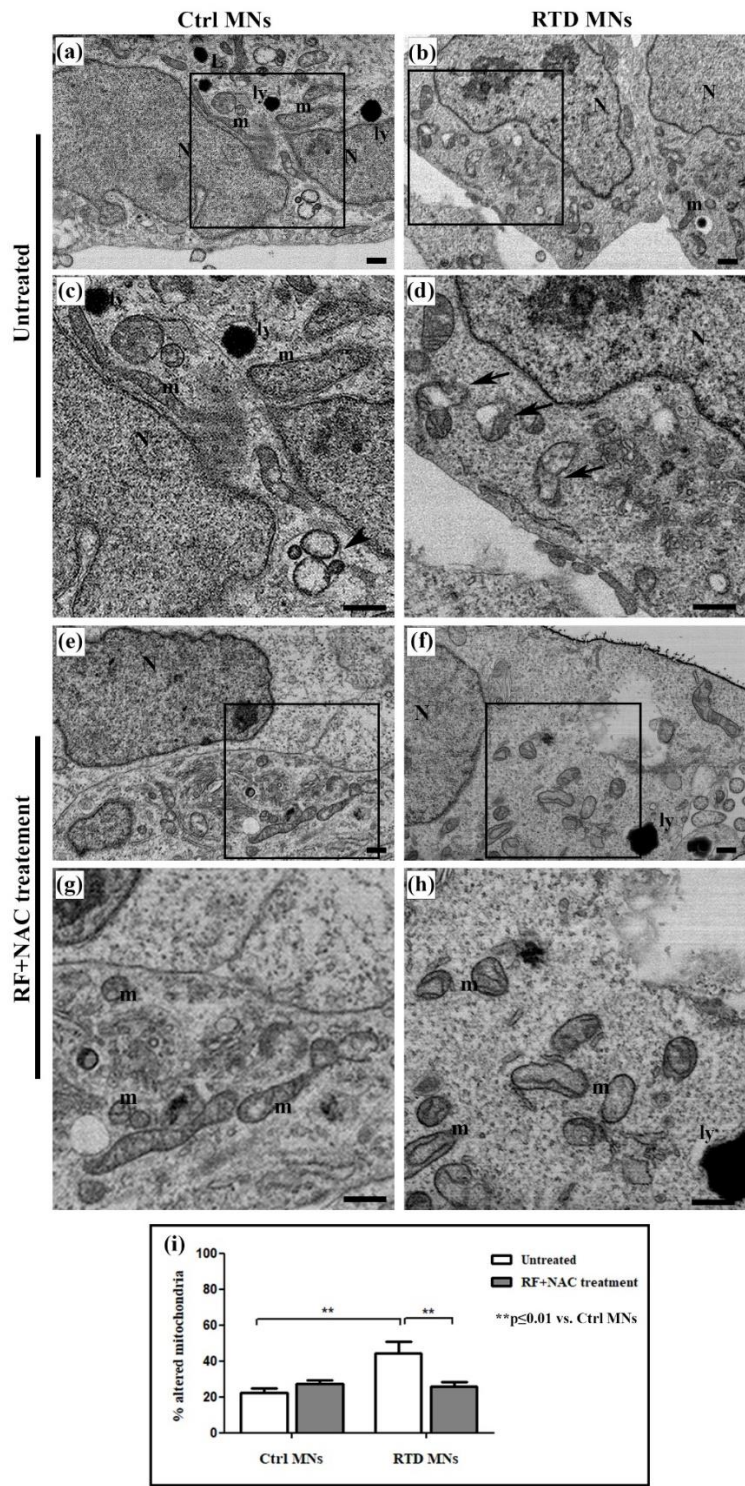
Figure 2. Ultrastructural analysis of RTD vs. Ctrl MNs, before and after RF+NAC treatment.

(a-d) FIB/SEM micrographs show the presence of several altered mitochondria in RTD MNs, with swollen morphology and deranged cristae (a, c), as opposed to regularly shaped organelles in Ctrl cells (b, d). After RF+NAC treatment (e-h), improved mitochondrial ultrastructure in RTD MNs is observed. (i) Statistical analysis demonstrates significantly higher number of damaged mitochondria in RTD MNs as compared to Ctrl. RF+NAC treatment restores mitochondrial morphology in RTD MNs (**$p \le 0.01$ vs. Ctrl MNs). Scale bars, 1μm. N, nuclei; m, regular mitochondria; ly, lysosomes; black arrows, altered mitochondria; black arrowheads, autophagosomes.

4. Discussion

Riboflavin (RF) and its derivatives, FMN and FAD, play a crucial role in essential cellular processes including mitochondrial energy metabolism, stress responses, vitamin and cofactor biogenesis, ensuring the catalytic activity and folding/stability of flavoenzymes [7, 12]. RF is therefore an indispensable nutrient for human health and represents one of the neglected antioxidants that may have an action independently or as a component of the glutathione redox cycle [13]. RF deficiency impacts on redox balance, compromising energy metabolism pathways and antioxidant defense mechanisms [7, 13]. Insufficient availability of the vitamin results in severe clinical conditions, particularly affecting MNs, thus sharing traits with amyotrophic lateral sclerosis (ALS) [14]. Emphasizing the importance of RF in human physiology and furthermore its efficient absorption and homeostasis are RTDs caused by recessive, biallelic mutations in the genes encoding human RF transporters [1]. Several *in vivo* and *in vitro* models have been generated to dissect the molecular mechanisms underlying RF deficiency [15-17], however a model recapitulating the human pathology is still lacking. Thus, we recently developed a patient-specific induced pluripotent stem cell (iPSC) model to study RTD phenotype from a morpho-functional viewpoint [9, 18].

In the present study, we took advantage of iPSC potential, to differentiate MNs. Hypothesizing the involvement of oxidative stress of mitochondrial origin in RTD MNs, we investigated whether the same alterations demonstrated in iPSCs derived from RTD patients [9], were maintained when undergoing differentiation protocol. This issue seemed crucial, in view of possible therapies targeting oxidative stress, particularly mitochondrial function, in RTD patients.

Overall, our data on patient specific MNs emphasize the involvement of mitochondrial alterations in RTD pathogenesis (Fig. 3). Indeed, significantly lower SOD2 immunofluorescence levels were detected in RTD vs. normal cells, revealing impaired mitochondrial functionality, with special reference to antioxidant properties. Notably, specific modulation of SOD2 in RTD condition is consistent with the reported role for RF, as an effective agent to boost the activity of this enzyme [19]. Such abnormalities in O_2^{-} scavenging ability, in total agreement with data obtained in undifferentiated cells from the same patients [9], likely result in exacerbated redox imbalance. This is however thought to be mainly contributed by defective energy metabolism pathways controlled by flavoproteins [7, 8]. Altered levels of antioxidant systems were also found in experimental dietary RF deficiency, supporting pleiotropic roles for RF in regulating redox balance, including gene expression regulation [15-17, 20-23]. Whatever its origin, oxidative stress may well account for the relatively poor development of neurites in RTD MNs, as revealed by βIII-TUB immunostaining. Patient-specific MNs display in fact shorter and fewer processes, as compared to their healthy counterparts, as suggested by a previous study [24].

Ultrastructural results well correlate with confocal data. Indeed, FIB/SEM analysis shows in RTD MNs remarkable mitochondrial dysmorphologies, strongly suggesting their dysfunction [9, 11]. Altered features associated to RTD condition include disrupted mitochondrial *cristae* and the presence of vacuoles in mitochondrial matrix. Since mitochondrial dysfunction is known to trigger mitophagy, as a quality control mechanism, we looked for autophagic vacuoles. However, RTD cells failed to show an appreciable number of double-membrane limited vacuoles, containing intact or partially digested organelles. Differently, in healthy MNs we detected numerous autophagosomes and lysosomes, suggesting active autophagic processes, as expected for an intensely active cell type. Possible involvement of insufficient/defective systems of repair/removal of damaged organelles in RTD pathogenesis has been suggested [8, 24], and certainly deserves further studies. However, our present and previous data still support mitochondrial-generated redox imbalance as responsible for the RTD altered phenotype.

We also explored novel therapeutical approaches by combining RF supplementation -known to partially ameliorate RTD phenotype- with the antioxidant, namely N-acetylcysteine (NAC), which is widely employed for free radical scavenging and glutathione replenishment. Indeed, this combined treatment proved successful, in improving patients' cell morphology, particularly as regards

mitochondrial ultrastructural features. This result is consistent with work in progress at our laboratories showing that such combined treatment results in increased neurites lengths in RTD MNs [25]. Studies on different pathological conditions have associated the efficacy of NAC with its action in cystine-glutamate exchange, glial glutamate transporters normalization, and in restoring glutamatergic tone on presynaptic receptors in reward regions of the brain [26-27]. Importantly, NAC has a long-established safety record and does not require titration to achieve the target dose [26], thus allowing to propose this compound for future therapeutic studies aimed at improving clinical outcomes of RTD patients.
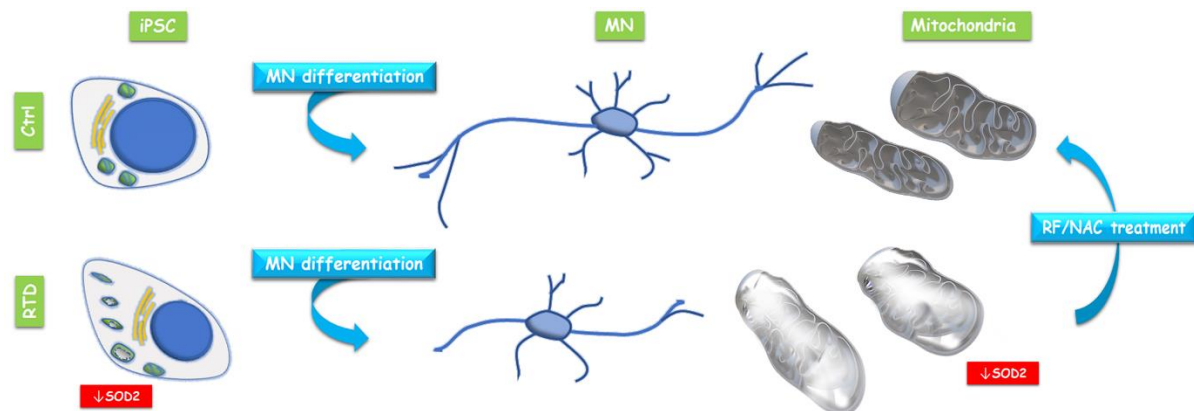

Figure 3. Conceptual schema showing mitochondrial alteration in RTD iPSCs and iPSC derived MNs and beneficial effects of RF+NAC treatment on mitochondrial morphology.

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Conflicts of Interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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